DETERMINATION OF 5α-ANDROST-16-EN-3-ONE CONCENTRATIONS IN BOAR SERUM AND FAT USING TIME-RESOLVED FLUOROIMMUNOASSAY

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INTRODUCTION

Intact (non-castrated) male pigs possess superior fattening performance and carcass traits compared with castrates and gilts. However, castration of male piglets is still a common practice in many countries because in a small proportion of intact male pigs the fat emits an unpleasant taint during cooking. One main compound associated with boar taint is androstenone (5α-androst-16-en-3-one), which is synthesized primarly in the testes, released into the blood and stored in the adipose tissue and salivary glands.
Immunological assays are well suited for the androstenone analysis, as they are simple and rapid to perform and offer the possibility of screening of a large number of samples. The aim of the current study was to develop a time-resolved fluoroimmunoassay for androstenone based on dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA®), and to obtain information regarding the level of androstenone in Finnish pigs.

METHODS

Reagents: The antiserum against androstenone 3-(O-carboxymethyl)oxime-BSA conjugate was kindly donated by Dr. Storm (Intervet International B.V., The Netherlands). Europium-labeled androstenone was prepared in two steps. Androstenone, sodium acetate and carboxymethoxylamine hemihydrochloride were refluxed in ethanol, followed by work-up to prepare androstenone 3-(O-carboxymethyl)oxime. The oxime was labeled with europium chelate using water soluble carbodiimide [1]. Labeled androstenone was purified by HPLC. Anti-rabbit Ig-coated microtitration wells, buffers and instrumentation were obtained from Wallac Oy (Finland).
Sample material: Blood samples (approx. 10 ml) were collected during exsanguination and allowed to clot for 1 h. Serum was then isolated by centrifugation and stored at -20 °C. Fat samples were collected from the neck region and stored at -20 °C. Serum and fat standards were prepared by spiking gilt or barrow samples with known amounts of androstenone.

Sample preparation: Fat sample was melted in a microwave oven or thermal block (60 °C) and 30 µl of liquid fat was transferred to a tube containing 500 µl of methanol. The sample was extracted for 30 min (55 °C) and vortex-mixed once during the incubation. A 50 µl aliquot of methanol extract was diluted 1:9 with DELFIA Assay Buffer and analyzed. Serum samples were diluted 1:6 with buffer and analyzed directly without extraction or purification.

Time-resolved fluoroimmunoassay: 50 µl of sample and 50 µl of europium-labeled androstenone in suitable dilution were pipetted into anti-rabbit -coated (secondary antibody) microtitration wells. 50 µl of diluted primary antibody was added and after incubation (1.5 h) at room temperature, the wells were washed six times with DELFIA Wash Solution. DELFIA Enhancement Solution (200 µl) was added and the wells were shaken for 5 minutes at room temperature. The enhanced fluorescence was measured in a 1234 DELFIA Fluorometer.

RESULTS AND DISCUSSION

The first immunological methods for androstenone analysis were based on radioimmunoassay [2-4] (RIA) and they involved the disadvantages connected with potentially hazardous radioactive materials. RIAs are nowadays largerly replaced by non-radioisotopic alternatives, especially enzyme-linked immunosorbent assay (ELISA). This trend is seen also in androstenone assays [5-9]. Enzymes used in the ELISA systems have the general advantage of a high signal amplification, but the signal is very much dependent on incubation time, temperature and other physical and chemical conditions during the substrate incubation. They are also sensitive to interfering substances in samples, such as endogenic enzymes or inhibitors.
Fluorescent labels offer an alternative in the field of non-radioisotopic detection but the sensitivity of the assay is limited by background interference. This interference can be avoided using time-resolved fluorometry. Some lanthanides, such as europium, form highly fluorescent chelates with certain organic ligands. The lanthanide fluorescence has an exceptionally large Stokes' shift (the difference between the excitation and emission wavelengths), narrow emission band, long decay time and high quantum yield. In DELFIA system, a nonfluorescent chelate is employed to bind europium to the analyte [10]. After the bioaffinity reaction is completed, the europium ions are dissociated from the chelates by means of an enhancement solution in which the lanthanide ions form new fluorescent complexes with β-diketone. Time-resolved fluorometry involves measurement of light at a fixed time after the fluorophore has been excited. By this time, background fluorescence has died away (Figure 1).
DELFIA showed good performance in the androstenone analysis. The assay sensitivity, defined as the mean signal of 10 replicates of the zero standard plus three times the standard deviation, was 0.8 ng/ml in serum analysis and 40 ng/g in fat analysis. The serum assay had a working range of 1-90 ng/ml and, on average, 103% of spiked androstenone was recovered. The average coefficients for intra- and interassay variation were 5.4% and 8.1%, respectively (3 samples, 10 replicas). Fat androstenone was measured in the range of

50-4000 ng/g (Figure 2) and the mean intra-/interassay variation coefficients were 6.4% (3 samples, n=10) and 8.2% (3 samples, n=7). The specificity of the antisera was assessed for related steroids (Table 1) and their influence on the assay results should be negligible. Androstenone concentrations in fat samples varied considerably between boars, and levels up to 4 µg/g were measured (median 471 ng/g, n = 57). Serum androstenone levels varied between 1.5 ng/ml and 110 ng/ml, the median being 15.2 ng/ml (n=61). In accordance with the results of Andresen [11] and Booth *et al.* [12], the serum levels above 15 ng/ml were always accompanied by a heavy accumulation of androstenone in adipose tissue and a significant correlation was found between boar serum and fat androstenone concentrations ($r = 0.70$, $p < 0.001$, $n = 51$) (Figure 3).

CONCLUSIONS

A simple and reliable time-resolved fluoroimmunoassay was developed for the estimation of androstenone levels in pigs. The method is well suited for routine screening of androstenone in boar meat.

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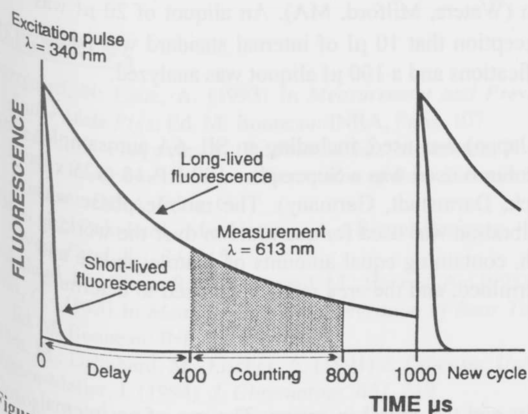

Figure 1 Principle of time-resolved fluorescence

Table 1. Assay cross-reactivity expressed as a percentage at 50% inhibition of maximum binding

compound	cross-reactivity (%)
5α-androst-16-en-3-one	100.0
5α-androst-16-en-3β-ol	10.4
5α-androst-16-en-3α-ol	6.3
4-androstene-3,17-dione	3.4
5α-androstan-17β-ol-3-one	0.5
5α-dihydrotestosterone	0.5
testosterone	0.4
17β-estradiol	<0.02

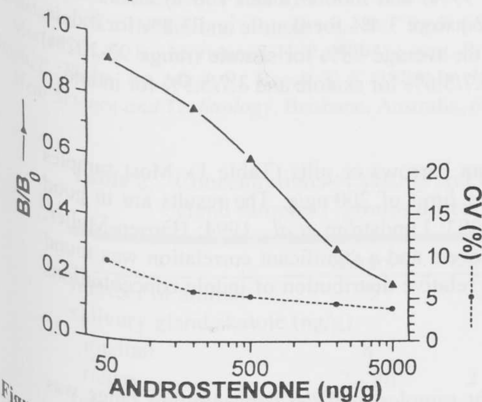

Figure 2 Standard curve and precision profile of fat androstenone assay (n = 10)

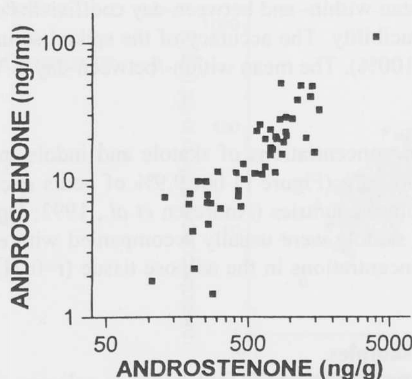

Figure 3 The relationship between fat and serum androstenone concentrations (n = 51)